

ALESSANDRA RUGGI*, ROBERTA CIMMARUTA*, GIANLUCA FORTI **
& GIUSEPPE NASCETTI*

PRELIMINARY STUDY OF A HYBRID ZONE BETWEEN
SPELEOMANTES ITALICUS (DUNN, 1923) AND
S. AMBROSII (LANZA, 1955) ON THE APUAN ALPS,
USING RFLP ANALYSIS

(AMPHIBIA, PLETHODONTIDAE)

INTRODUCTION

In the last twenty years the study of hybrid zones has been increased and dealt successfully (ARNOLD 1992). Hybrid zones are “natural laboratories” to investigate the evolution of the reproductive isolation mechanisms in geographically isolated taxa that subsequently met and hybridized in a secondary contact (BARTON & HEWITT 1985; HARRISON 1990; HEWITT 1993). Since the glacial/interglacial stages, characterising the Quaternary epoch, have been relevant in determining isolation/secondary contact events, it has been hypothesized that the hybrid zones in the Alps and Pyrenees could have been formed during the last 9000 years (HEWITT 1988).

The DNA markers have been very useful in describing subspecific divergence and geographical species subdivision (AVISE 1998). In particular, mitochondrial DNA is suitable for studies concerning molecular evolution, hybrid zones, phylogeny and colonization events. This is due mainly to its molecular (WILSON *et alii* 1985) and inheritance properties (GYLLENSTEN *et alii* 1985; AVISE 1998). The rate of divergence of the mtDNA is 5-10 times faster than

* Dipartimento di Ecologia per lo Sviluppo Economico Sostenibile, Università della Tuscia, Via S. Giovanni Decollato 1, 01100 Viterbo, Italia;
e-mail: alessandra.ruggi@virgilio.it, cimmaruta@unitus.it & nascetti@unitus.it

** Museo del Fiore, Piazza G. Fabrizio 17, 01021 Acquapendente (VT), Italia;
e-mail: museo.fiore@tin.it

nuclear DNA (BROWN *et alii* 1979); it's fast enough to show population differences across a species range, and slow enough to skip saturation problems over a few million years. There is also a substantial difference in the evolutionary rates of mitochondrial DNA in different taxa (MORITZ *et alii* 1987). In the study of hybrid zones the mtDNA allows to investigate the direction of the gene flow and to verify if it is sex-biased or not (AVISE & SAUNDERS 1984; CARR *et alii* 1986; NELSON *et alii* 1987; SZYMURA *et alii* 1985; TAYLOR & HERBERT 1993), allowing ecological and ethological inferences. In the Plethodontid family a few hybrid zones have been investigated so far (HAIRSTON *et alii* 1992; YANEV 1980; WYNN 1986; WAKE *et alii* 1980; KARLIN & GUTTMANN 1981), and this is the only one found in the genus *Speleomantes*.

MATERIAL AND METHODS

Two hundred and five specimens were processed from 22 populations, collected between 1980 and 1993 (tab. 1, fig.1). Allozyme electrophoresis was used in a previous study to assess the genotype of each sampled individual (*S. italicus*, *S. a. bianchii* or introgressed) (CIMMARUTA 1993). From the same electrophoretically characterised specimens the total genomic DNA was extracted from frozen muscle tissue following the CTAB/phenol-chlorophorm protocol (MURRAY & THOMPSON 1980).

The double strand PCR amplification (SAIKI *et alii* 1988) was performed in a 50 µl volume containing: 5-20 ng of total purified genomic DNA; 2.5 u of Taq DNA polymerase, the primers (0.2 µM), MgCl₂ (2.5 mM), 1µl dNTP (0.2 mM). The amplification was carried out using GeneAmp PCR System 2400 (Perkin Elmer), and began with an initial denaturation at 94°C for 5 minutes. Thermocycling parameters were: denaturation 94°C for 1', annealing at 45°C for 30'', extension at 72°C for 1', for 35 cycles. The reaction ended at 72°C for 10'. This yielded 501-bp-sized fragment, a part of the cytochrome-b region, corresponding to position 16,580 (5'end) and 17,078 (3'end) of *Xenopus laevis* (ROE *et alii.* 1985). The following primers were used for double strand PCR amplification: MVZ25-L (MORITZ *et alii.* 1992) modified into HY01 (5'-AAAGAAACTT-GAAATATTGGAGT-3') and MVZ16 (5' - AAATAGGAAWTAT-

CAYTCTGGTTTWTAT- 3'), designed to match plethodontid salamanders (MORITZ *et alii* 1992).

Table 1 - Sampling list of populations, with the collection locality and province. The cadastral number of the caves is also reported, when available.

No.	Population name and locality	Abbreviation
1	Pulica (Tana della Bastiola 481T/MS), Massa Carrara	APU
2	Tana di Bedizzano (130T/MS), Massa Carrara	ABE
3	Forno (Buca della Renella 272T/MS and Buca della Cava dell'Onice 287T/MS), Massa Carrara	AFO
4	Near Porneta, Massa Carrara	APO
5	Pian della Fioba (botanic garden), Massa Carrara	APF
6	Near Galleria di Tecchia, Massa Carrara	ATE
7	Near Galleria Valsora, Massa Carrara	AVS
8	Near Galleria M.te Pelato, Lucca	AMP
9	Within Campagrina built up area, Lucca	ACM
10	Near Cave Henraux, Lucca	ACH
11	Near Isolasantà, Lucca	IIS
12	Near Arni, Lucca	AMU
13	Near Galleria del Cipollaio, Lucca	ICI
14	Near Vagli di Sotto, Lucca	IVA
15	Magnano (Tana del Pollone, no cadastral number), Lucca	IMN
16	Piteccio (Buca di Nadia 732T/PT, Buca di Anna 829T/PT, Buca delle Fate di Calabianna 830T/PT), Pistoia	IPT
17*	Calvana Mt. (Buca del Castagno 602T/FI), Florence	ICA*
18	Near Cardoso Stazzemese, Lucca	ICS
19	Borgo a Mozzano (G. della Cartiera 833T/LU), Lucca	IBM
20*	Near S.Giovanni in Petroio (Buca Ghiandaia 736T/FI, Buca delle fate di casa Ferrucci 192T/FI), Florence	IFI*
21	S. Martino in Freddana (Buca delle Fate 225T/LU), Lucca	ISM
22*	Buca Tana di Maggiano (827T/LU), Lucca	IMA*

* These samples have been studied using allozymes only, not RFLPs on mtDNA.

Sequencing was performed using A.B.I. Prisma 310 sequencer (Perkin Elmer) containing 2 μ l of ABI-mix for sequencing (Perkin Elmer), 1 μ l of primer (3.2 pmol) and about 50 ng of PCR product. The primers for sequencing were the same as PCR amplification.

Mitochondrial DNA (mtDNA) sequences of a double strand were checked using Chromas program version 1.6 (Technelysium Pty Ltd, 1998-2000) and aligned using the Clustal X program version 1.8 (THOMPSON *et alii* 1997). The suitable set of endonuclease enzymes providing specific haplotypes, was obtained using the program DNA for Windows version 2.2 (DIXON 1999). The chosen set of restriction enzymes is the following: HaeIII, SspI, RsaI, MboI. Enzymatic digestions were performed in a total volume of 10 μ l containing 7.5 μ l of the PCR product; 1 μ g of BSA and 5u of endonuclease. The digested DNA fragments were run on agarose 3% gel.

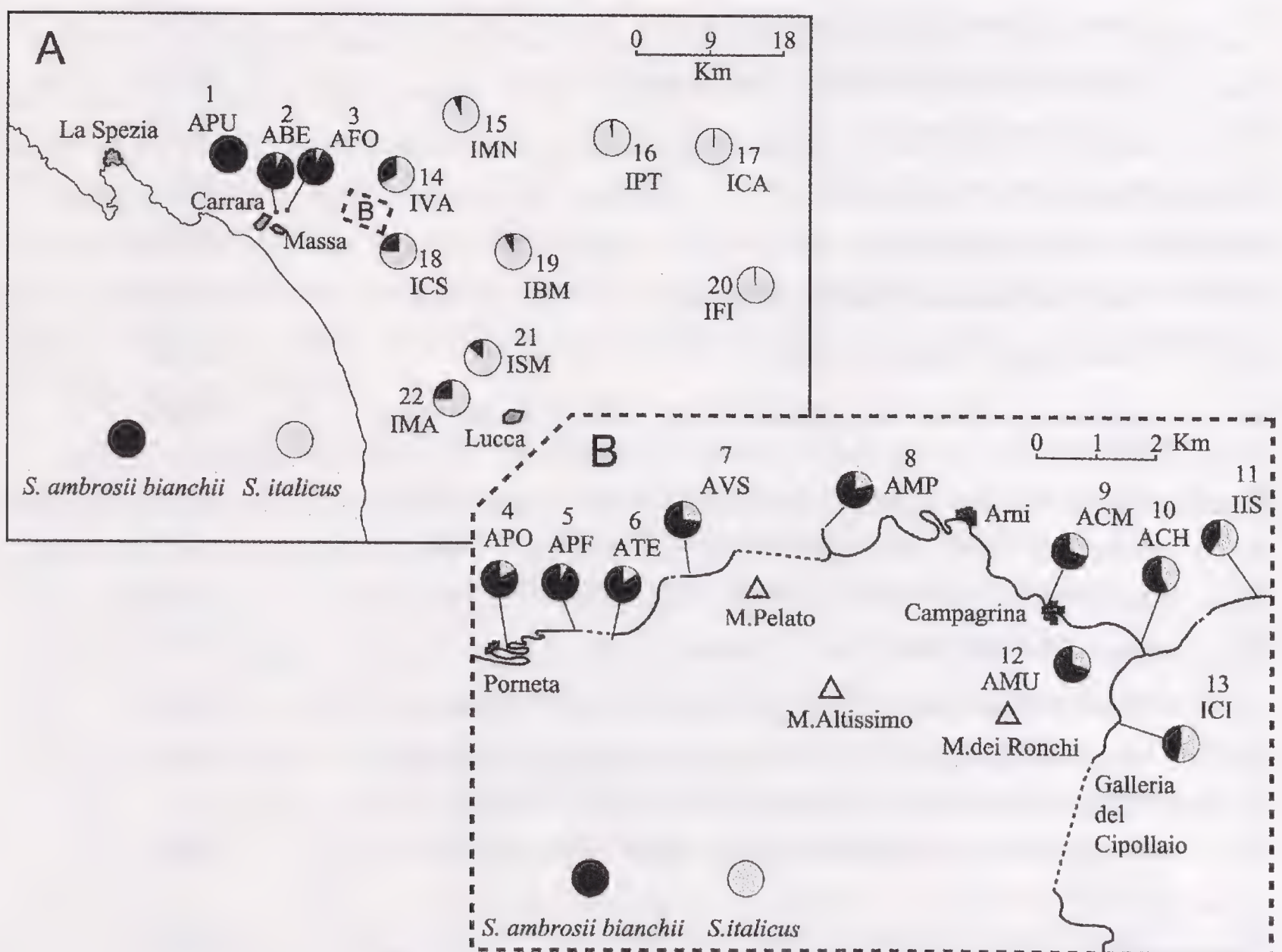


Fig. 1 - Mean allele frequencies of the samples of *S. a. bianchii* and *S. italicus*. B is the area of the contact zone enlarged (From CIMMARUTA 1993, modified).

Table 2 - Haplotypes observed in the studied taxa and in the samples from the contact zone (A1, AP1, AP2, H1). Different endonuclease digestion patterns referring to each enzyme are shown (A, B, C, D).

Restriction Enzyme	HaeIII	SspI	RsaI	MboI	Haplotype
<i>S. a. ambrosii</i>	B	B	B	B	A1
<i>S. a. bianchii</i> (Western populations)	B	C	A	A	AP1
<i>S. a. bianchii</i> (Eastern populations) and contact zone	B	D	A	B	AP2
<i>S. italicus</i> and contact zone	A	A	A	A	H1

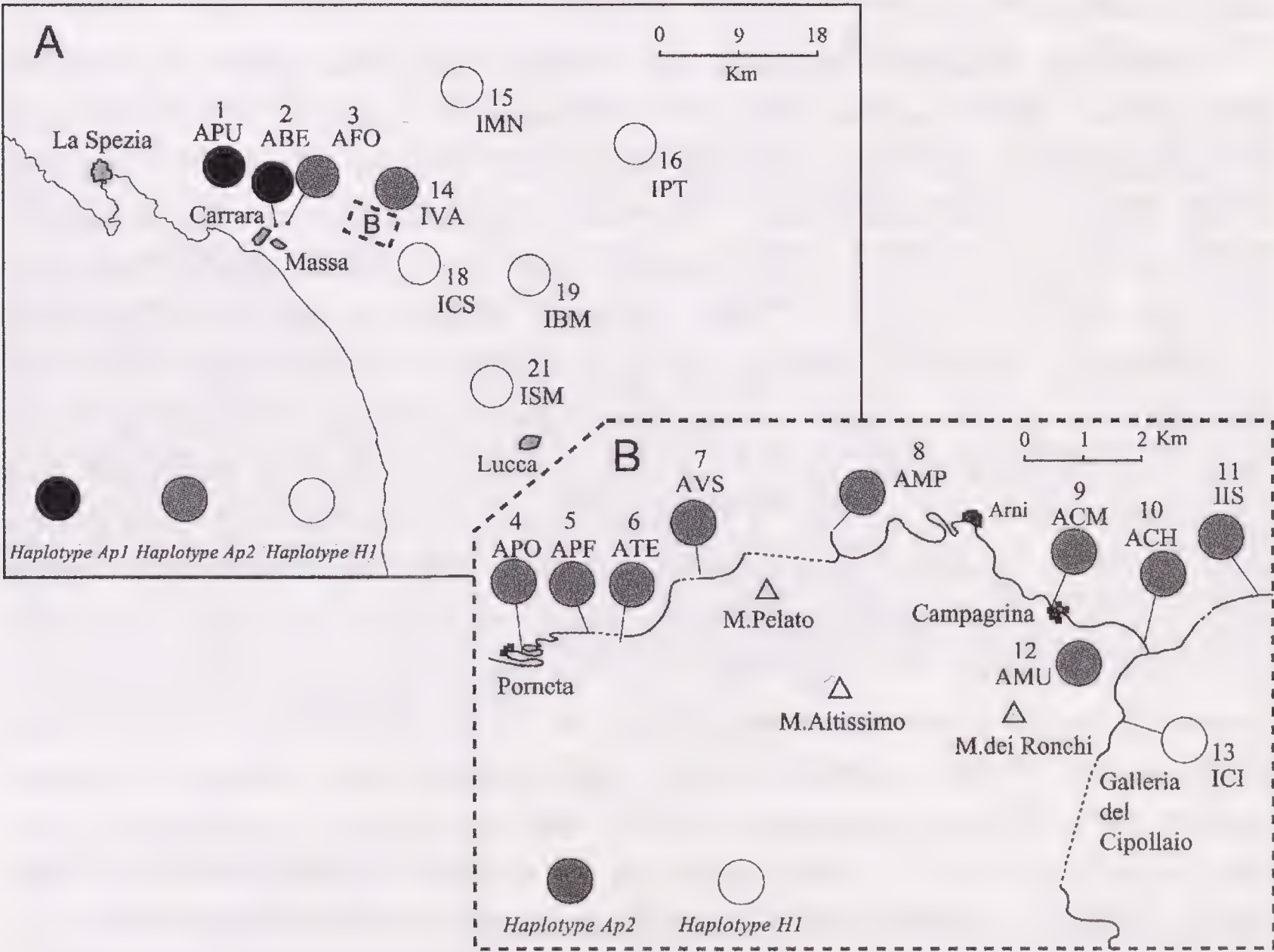


Fig. 2 - The haplotypes found in the studied samples of *S. a. bianchii* and *S. italicus* (A) and from the contact zone (B).

RESULTS

Full sequences of the cytochrome b 501-bp-sized fragment have been obtained from an overall number of 17 specimens of *S. a. ambrosii*, *S. a. bianchii*, *S. italicus* and from the contact zone. These sequences have been used to infer the restriction patterns and the corresponding haplotypes per each studied population (tab. 2).

The obtained haplotype frequencies per sample are shown in fig. 2. The observed pattern show that *S. a. bianchii* has two different haplotypes, observed in different populations. The two western populations, APU and ABE, have the same fixed haplotype (AP1), while the nearby sample from eastern population, AFO, is fixed for a different one (AP2). Interestingly, only this last haplotype (AP2) is hold by all the samples from the contact zone (n. 4-12 and n. 14; fig. 2). Southern populations have the *S. italicus* typical haplotype (H1) (n.13 and n. 15-16-18-19-21).

DISCUSSION

Previous studies, carried out using allozyme electrophoresis, have shown that the two taxa *S. a. bianchii* (LANZA *et alii* 2005) and *S. italicus* have met and hybridized in the Apuan Alps (CIMMARUTA 1993; NASCETTI *et alii* 1996). The two taxa have discriminant alleles fixed at 8 out of the 31 loci studied and their mean allele frequencies are shown in Fig. 1. The pattern observed for the allozymes is strikingly different respect to that obtained from mtDNA. The populations in the contact zone have alleles typical of both species, with frequencies close to 1:1 in many samples, as for example the populations n. 9-13 in the “Cipollaio” area where the two taxa probably came in contact and hybridized (fig. 1B). In the contact zone only recombinant genotypes have been recorded, without parental specimens neither F1 hybrids. On the contrary, the haplotype frequencies never show the coexistence of *S. a. bianchii* and *S. italicus* haplotypes: all the samples from the contact zone have the same haplotype (AP2) as population AFO. An exception is represented by the population n. 13 (ICI), having *S. italicus* haplotype (H1) (fig. 2B) in spite it is within the hybrid zone boundaries, as identified by allozyme markers. This shows another difference between nuclear and mitochondrial patterns: allozymes show an asymmetric intro-

gression, with *S. a. bianchii* alleles strongly entering into *S. italicus* gene pool, far away from the contact zone (fig. 1A), while mtDNA shows a clean-cut subdivision of the AP2/H1 haplotypes, both unable to cross the borderline represented by the “Cipollaio” area (fig. 2B).

Differences in the patterns of nuclear vs. mitochondrial markers are frequently observed in hybrid zones, due to their different features. In particular, being mtDNA maternally inherited, its distribution pattern is strongly dependent from sex-biased dispersal and is more subject to genetic drift effects, because of the smaller effective population size (one quarter of total N_e). These properties, together with the higher evolutionary rate of mtDNA respect to nuclear DNA, make mtDNA able to retain the marks of recent paleogeographic events and can explain the higher fragmentation observed within *S. a. bianchii* using mitochondrial markers: two different haplotypes characterise western (n. 1-2) vs. eastern (n. 3) populations, a difference not highlighted using allozymes.

In conclusion, the comparison of nuclear vs. mitochondrial markers suggested a biogeographic scenario originating the present pattern of hybridization between *S. a. bianchii* and *S. italicus*. The genetic subdivision of *S. a. bianchii* could be a legacy of ice ages, that fragmented *Speleomantes* populations in many refugia (FORTI *et alii* 1998; CIMMARUTA *et alii* 2005). Mitochondrial genes could retain stronger signs of this recent history because of their above mentioned features, indicating the existence of more than a single remnant nucleus in the Apuan Alps, as suggested by the presence of two different haplotypes (AP1/AP2) in the area. The further expansion during interglacials could have led to the meeting of specimens bearing western-*bianchii* haplotype (AP2) with *S. italicus* (H1). The hybridization occurred was probably due to *S. a. bianchii* males entering *S. italicus* range, as suggested by the presence of *bianchii* nuclear genes into *S. italicus* populations not having *bianchii* mtDNA in their gene pool.

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ABSTRACT

It has been recently demonstrated that the species *Speleomantes ambrosii* includes two geographically and genetically distinct forms: *S. ambrosii ambrosii*, from the area of La Spezia on the hydrographic right side of the Magra river, and *S. ambrosii bianchii*, ranging from Massa Carrara to the west side of the Apuan Alps. In this area *S. a. bianchii* and *S. italicus* came in contact and hybridized, as shown by previous allozyme analysis.

Within this study a RFLP protocol has been set up to investigate the mitochondrial genome of *Speleomantes* species. The fragment amplified is 501-bp-sized of Cytochrome b region. The specific haplotypes characterising the taxa *S. italicus*, *S. a. ambrosii* and *S. a. bianchii* have been obtained using a set of four restriction enzymes. The haplotypic pattern evidenced is not coincident with the allozyme one, showing a high genetic fragmentation of *S. a. bianchii* and a segregation of *bianchii-italicus* haplotypes in the contact zone.

RIASSUNTO

Studio preliminare di una zona di ibridazione tra *Speleomantes italicus* e *S. ambrosii* sulle Alpi Apuane, utilizzando l'analisi RFLP (Amphibia, Plethodontidae).

La specie *Speleomantes ambrosii* presenta due forme geneticamente differenziate: *S. ambrosii ambrosii*, in provincia di La Spezia, alla destra idrografica del fiume Magra, e *S. ambrosii bianchii* in provincia di Massa Carrara fino al versante occidentale delle Alpi Apuane, dove quest'ultimo è venuto in contatto ed ha ibridato con *S. italicus*. In questo studio preliminare è stata messa a punto la tecnica RFLP su DNA mitocondriale di *Speleomantes* e si è analizzata una porzione di mtDNA di 501 bp codificante per il Citocromo b. Sono stati individuati gli aplotipi caratteristici di *S. italicus*, *S. a. ambrosii* e *S. a. bianchii*.

I risultati ottenuti mostrano un pattern aplotipico non coincidente con quello allozimico, ottenuto in studi precedenti. Il confronto fra le due serie di dati ha permesso di evidenziare una considerevole frammentazione genetica di *S. a. bianchii* e la separazione degli aplotipi di *S. a. bianchii* e *S. italicus*, che non sono mai presenti contemporaneamente nella stessa popolazione.